REMARKS

Election/Restriction

The Examiner has maintained the restriction of the claims and has withdrawn non-elected claims 3, 4, 7, 8, 10, 13-17, 22, 23 and 25-28 from examination. The non-elected claims have been cancelled and will be pursued in one or more divisional applications at Applicants' discretion.

Claim Objections

Claims 5, 6, 11, 12, and 24 have been objected to for depending from non-elected claims or for reciting non-elected subject matter. Claim 21 has been further objected to for a typographical error. The claims have been amended as indicated above, to delete any dependence from non-elected claims or recitation of non-elected subject matter. In addition, claim 21 has been amended to correct the typographical error.

Rejections under 35 U.S.C.§112, 2nd paragraph

Claims 1, 2, 6, 9, 12, 19, 20 and 21 have been rejected under 35 U.S.C.§112, 2nd paragraph for the reasons discussed on page 4, spanning page 5 of the Office Action. These issues have been addressed with the above-indicated amendments.

Specifically, claims 1, 2 and 9 have been rejected for recitation of "antibody or its fragment." Applicants note that "its fragment" refers to the antibody and have amended the claim accordingly.

Claims 2 and 9 have been rejected for failing to define stringent hybridization conditions. Claims 2 and 9 have been amended to incorporate the hybridization conditions of page 18 of the specification.

Claims 6 and 12 have been rejected as being confusing in the recitation of "mouse-derived gene". The claims have been amended to recite "mouse gene", to clarify the claims.

Claim 19 has been rejected for recitation of "transformant". Claim 19 has been amended to more clearly be directed to "transformed cells."

Claim 20 has been rejected for recitation of "such as a macrophage." The indicated subject matter has been deleted from claim 20 and presented as new claim 29.

Finally, Applicants have amended claim 21 to be in more proper format.

Rejection Under 35 U.S.C.§101

Claims 1, 2, 5, 6, 9, 11, 12 and 20 have been rejected as being drawn to a product of nature. Claims 1, 2 and 5 have been

amended to recite "isolated gene" and claims 11, 12 and 20 to recite "purified protein" or "purified receptor" as appropriate.

Rejection under 35 U.S.C.§§101 and 112, 1st paragraph

On page 5, spanning page 6 of the Office Action, the Examiner rejects the claims under 35 U.S.C§§101 and 112, 1st paragraph for an asserted lack of utility. The Examiner bases this rejection on the fact that the specification references Japanese application No. 9-266591 and the corresponding Japanese publication in support of the antibodies recognizing a protein that induces G-CSF. However, there are no English language equivalents of these documents for the Examiner to evaluate.

Applicants traverse this rejection and withdrawal thereof is respectfully requested. Attached hereto is an English abstract of JP-9-266591. From the English abstract of JP '591 it is evident that the inventors in JP '591 obtained an antibody that has activity for inducing G-CSF. If the Examiner would like a full English translation of JP '591, she is requested to please contact the undersigned. In addition, to the English Abstract of JP '591, also attached hereto is the journal article by Aoki et al.; J. Leukocyte Biology **68**:757 (2000). (Yoshiko Aoki is an inventor of the subject matter of JP '591 and of the present invention.) The

also attached hereto is the journal article by Aoki et al.; J. Leukocyte Biology **68**:757 (2000). (Yoshiko Aoki is an inventor of the subject matter of JP '591 and of the present invention.) The Aoki et al. article also discloses an antibody that has G-CSF inducing activity.

The protein of the present invention has the feature of binding to G-CSF inducing antibodies or antibody fragments. See, for example, feature (b) of claims 1 and 9. Thus, the protein of the invention may also act at the entry point of induction and stimulation of G-CSF. As disclosed on page 42, lines 11-21 of the specification, the protein of the invention may be a receptor that acts as a G-CSF inducer. The protein of the invention, can therefore, be used to screen for a substance that can induce G-CSF or other substances. See page 43, line 6 to page 45, line 21 of the specification. The specification also teaches that the protein, nucleic acid etc. of the invention may be used as a pharmaceutical agent (see, for example, page 48, line 17 to page 51, line 1). As such, Applicants have adequately and sufficiently disclosed credible, specific and substantial asserted utilities for the present invention. Withdrawal of the rejection is therefore respectfully requested.

The Examiner further asserts that the specification is not enabled for producing all of the variants of SEQ ID NO:2 that are encompassed by the claims. This issue is further addressed below along with the rejection under 35 U.S.C. §112, 1st paragraph for lack of written description.

Rejections under 35 U.S.C.§112, 1st paragraph - Written Description and lack of enablement

The Examiner rejects the claims under 35 U.S.C. §112, 1st paragraph for a lack of enablement and for lack of written description with the assertion that the specification does not describe or enable the scope of variants encompassed by the claims. The Examiner states that the specification does not provide any description of what domains of the protein are important and must be conserved.

The present specification discloses that the protein of SEQ ID NO:2 is "thought to be a membrane glycoprotein with one membrane-spanning domain, which comprises an extracellular portion of 107 amino acids, a membrane-spanning portion of 23 amino acids and an intracellular portion of 111 amino acids". See, page 55, lines 23-27 of the specification.

Type I membrane proteins are membrane-spanning proteins with the N-terminus being an extracellular domain. Thus, amino acids 1-107 of SEQ ID NO:2 are the extracellular domain, amino acids 108-130 are the membrane spanning domain, and amino acids 131-241 are an intracellular domain. Thus, the present protein is structurally well-characterized. However, in an effort to facilitate the examination of the application, the claims have been amended to recite,

> a protein having **at least 80% identity with** the amino acid sequence listed as SEQ ID NO:2 of the Sequence Listing **through the conservative substitution of one or more amino acids**

Support for the amendments to the claims may found on page 20 of the specification. In addition, new claims 30-33 have been added, which recite either 90% or 95% sequence identity through the conservative substitution of one or more amino acids. Applicants believe that the amended claims and new claims 30-33 are fully supported and enabled by the specification. As such, allowance thereof is respectfully requested.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact MaryAnne Armstrong, PhD (Reg. No. 40,069) at the telephone number of the undersigned below, to conduct an

interview in an effort to expedite prosecution in connection with the present application.

Applicants request a three (3) month extension of time for filing the present response. The required fee is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____
Gerald M. Murphy, Jr., #28,977

MaryAnne Armstrong, PhD #40,069

P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

GMM/MAA/
0230-0169P

Attachments: English Abstract of JP '591;
Aoki et al.; J. Leukocyte Biology **68**:757 (2000)

Selective stimulation of G-CSF gene expression in macrophages by a stimulatory monoclonal antibody as detected by a luciferase reporter gene assay

Yoshiko Aoki, Shiken Sha, Hidehito Mukai, and Yoshisuke Nishi

Laboratory of Life Science & Biomolecular Engineering, Japan Tobacco Inc., Yokohama, Kanagawa, Japan

Abstract: We have identified a stimulatory monoclonal antibody (mAb) from autoimmune mice that selectively stimulates granulocyte colony-stimulating factor (G-CSF) gene expression in a mouse macrophage cell line. The induction was observed not only in the cell line, but also in normal peritoneal macrophages. This mAb bound to the monocyte/macrophage cell lines and pre-B leukemia cell lines, but also in normal peritoneal macrophages, whereas it did not bind to normal T and B cells in the spleen or fibroblastic cell lines. It could even bind to a human promyelocytic leukemia cell line, when they were differentiated into monocytic cells. On Western blotting, this mAb mainly recognized an approximately 30-kDa band and it was unique because there have been no reports of membrane-associated proteins with a similar molecular mass found in macrophages. These results suggest that there could be a specific gateway molecule to induce G-CSF in macrophages. *J. Leukoc. Biol.* 68: 757–764; 2000.

Key Words: RAW264.7 · peritoneal exudate macrophages

INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is one of cytokines that stimulate and activate segmented neutrophils in bone marrow and in the periphery. G-CSF is known to be produced mainly by monocytes/macrophages, and also by T lymphocytes and endothelial and stromal cells [1–3]. In animal models of chemotherapy and radiotherapy, G-CSF accelerates the recovery of neutrophils [4–6]. Administration of recombinant human G-CSF has shown marked efficacy in recovering neutrophils in cancer patients suffering from neutrophil agranulocytosis due to chemotherapy or radiotherapy, and in aplastic anemia patients after bone marrow transplantation [3, 7]. G-CSF has an advantage over other cytokines for therapeutic use because it has a selective activity to proliferate only neutrophils.

In stromal cells, fibroblasts, and endothelial cells, G-CSF is stimulated by several soluble factors including lipopolysaccharide (LPS) [8] and cytokines, such as interleukin (IL)-1 [9], IL-17 [10], tumor necrosis factor (TNF) [11], and oncostatin M [12], and by cellular contact with other cells [13, 14]. G-CSF is induced in monocytes/macrophages by soluble factors, such as LPS [15, 16], IL-1 [17], IL-3 [18], IL-4 [19], M-CSF [20], interferon-γ (IFN-γ) [21], and also cell-to-matrix interactions [22]. We previously showed that G-CSF was induced in the mouse macrophage cell line RAW264.7 by contact with extracellular matrix molecules, such as fibronectin (FN), vitronectin (VN), and laminin, and one of the RGD peptides, which are recognized by FN and VN receptors, i.e., integrins [23]. FN-mediated G-CSF induction was blocked by adding neutralizing antibodies (Abs) against integrin molecules to the medium. In addition, contact of this macrophage cell line with a pre-B leukemia cell line, NFS-60, induced G-CSF in a cell number-dependent fashion. These results suggested that there could be one or more cell-mediated signal pathways through which G-CSF is induced in macrophage cell lines.

During the course of our study, Fine et al. identified a small-molecular-weight compound that selectively induced G-CSF in human peripheral mononuclear cells, but it did not induce other cytokines or colony-stimulating factors (CSFs) with hemato-regulatory activity [24]. Their results raised the possibility of a signal pathway specific for G-CSF induction in macrophages. These results and our own results inspired us to determine whether there are specific gateway molecules that selectively transmit intracellular signals to induce G-CSF in macrophages. Most of our efforts have focused on the production of Abs that stimulate G-CSF gene expression. Some mAbs have been reported to support or inhibit hematopoiesis through interactions with CD18, VLA-4, or other unknown antigens [25–29]. In contrast, no mAbs have been reported to induce cytokine production in macrophages. In the present work, we found a cell membrane-associated gateway molecule that is uniquely recognized by a stimulatory antibody through which expression of G-CSF gene was triggered in a macrophage cell line. In this study we used a sensitive assay system using luciferase as a reporter for G-CSF and an autoimmune mouse as the source of monoclonal antibodies (mAbs).

Correspondence: Yoshisuke Nishi, Ph.D., Director, Laboratory of Life Science & Biomolecular Engineering, Japan Tobacco, Inc., 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan. E-mail: yoshisuke.nishi@ims.jti.co.jp

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Animals and cells

For immunization, 8- to 12-week-old inbred female MRL/MPJ-*lpr/lpr* mice were purchased from Charles River (Atsugi, Japan). For preparation of normal peritoneal exudate cells from the peritoneal cavity, we used 7- to 8-week-old outbred male ICR mice and inbred male C57BL/6 mice (both from Charles River).

For reverse transcriptase-polymerase chain reaction (RT-PCR), we used outbred male ICR mice because they gave a higher yield of peritoneal exudate cells. For a cytofluorometric analysis, we used inbred male C57BL/6 mice, because this strain is a type B haplotype, and the secondary antibody we used was against IgM^a, which is cross-reactive with MRL/MPJ-*lpr/lpr* haplotype and not cross-reactive with surface IgM on splenic B cells from C57BL/6 mice. The hypoxanthine-aminopterin-thymidine (HAT)-selective nonsecretory murine myeloma cell line PAI, which is used for making hybridomas, was obtained from the Japanese Collection of Research Bioresources (Tokyo). It was maintained in ASF104 medium (Ajinomoto, Tokyo) supplemented with 10% fetal bovine serum (FBS, Bio-Whittaker, Walkersville, MD), 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO-BRL, Rockville, MD). The mouse macrophage cell line RAW264.7 obtained from the American Type Culture Collection (Manassas, VA) was maintained in Eagle's minimal essential medium (EMEM; GIBCO) supplemented with 10% FBS plus nonessential amino acids (GIBCO) at 37°C under 5% CO_2 in humidified air.

Reagents and treatment

Mouse FN and VN were obtained from GIBCO. LPS from *Escherichia coli* 026:B6 was from Sigma (St. Louis, MO). For the cytofluorometric analysis, we used phycoerythrin (PE)-anti-mouse IgM^a, fluorescein isothiocyanate (FITC)-anti-CD14, FITC-anti-CD45, and FITC-anti-CD8 obtained from PharMingen (San Diego, CA) and a cell sorter EPICS ALTRA (Beckman-Coulter, Fullerton, CA). For detection of induced levels of TNF-α, IL-1α, IL-1β, and IL-6, cytokine kits from Endogen (Cambridge, MA) were used. Treatments were done in 96-well microtiter plates (Falcon, Oxnard, CA). For treatment with extracellular matrix (ECM) proteins, substratum-coated plates were prepared by incubating the plates with ECM proteins dissolved in phosphate-buffered saline (PBS) at room temperature for 2 h followed by rinsing twice with PBS. For treatment with LPS, it was dissolved in PBS.

Immunization, preparation of hybridomas, and antibody purification

MRL/MPJ-*lpr/lpr* mice were injected intraperitoneally with RAW264.7 cells at 2×10^7 cells/mouse in 0.5 mL of PBS and boosted twice with an equal number of the cells at 14-day intervals. Three days after the final boost, spleens were excised and splenocytes were harvested. The splenocytes were fused with the PAI cells following the standard protocol [30]. Briefly, fusions were performed using 50% polyethylene glycol 1500 (w/v) in 75 mM HEPES (pH8.0) (Roche Diagnostics, Mannheim, Germany) at a spleen cell-to-myeloma cell ratio of 8:1. Hybrids were plated in 96-well microtiter plates at a concentration of 10^5 viable cells/well in ASF104 medium supplemented with HAT (Roche Diagnostics). The medium was replaced every 3–4 days and aminopterin was removed after 21 days. Hybridomas were then subcloned by limiting dilution in the culture medium containing hypoxanthine and thymidine. For preparation of the antibodies, the cloned hybridoma cells at 2×10^6 cells were cultured in 500 mL serum-free ASF104 medium for 5 days. The mAbs were purified from the culture supernatants by the euglobulin precipitation method [31]. The mAbs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples with >95% purity were used for further analyses.

ELISA assays

RAW264.7 cells were used to determine antibody binding to cell surfaces. The cells were plated at 5×10^4 cells/well on flat-bottomed 96-well microtiter plates (Falcon) and cultured overnight. Supernatants from the hybridomas were added, and then incubated for 60 min at room temperature. The plates were washed twice with PBS and horseradish peroxidase (HRP)-anti-mouse Igs (Zymed Laboratories, South San Francisco, CA) were added and incubated for 30 min. Peroxidase was detected with 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) and read on a spectrophotometer (M-Tmax, Molecular Dynamics, Uppsala, Sweden) at 405 nm. In some cases, the RAW264.7 cells were fixed with 0.25% glutaraldehyde for 5 min and washed thoroughly with PBS. Nonreactive sites were then blocked with 1.0% gelatin. Plates were washed and blocked with 1.0% gelatin again. In other cases, the RAW264.7 cells were washed twice with PBS, lysed with buffer containing 0.5% Igepal CA-630 [an equivalent product for Nonidet P-40, (octylphenoxy)-polyethoxyethanol, Sigma] and 0.1% EDTA at 4°C for 1 h, and centrifuged at 14,000 *g*. The supernatant was then used to coat flat-bottomed 96-well microtiter plates at 5 μg protein/well.

Measurements of G-CSF induction using a luciferase reporter gene

G-CSF activity was measured quantitatively using the cloned RAW264.7 cell line with the mouse G-CSF promoter gene linked to the luciferase gene as a reporter [29]. As a promoter of G-CSF gene, we used a 1.7-kb upstream region from the translation-initiation point of G-CSF gene [32]. This sequence was screened from a mouse genomic library, digested with *Xho*I and *Nco*I and inserted into a multiple cloning site of Picagene Enhancer Vector 2 (Wako, Osaka, Japan). Then, neomycin-resistant gene was cut off from the pMC1NeoPolyA 3854 (Stratagene, La Jolla, CA) at restriction sites of *Xho*I and *Sal*I and inserted into the *Sal*I site, which is located just downstream from an SV40 enhancer of Picagene Enhancer Vector 2 with the G-CSF promoter region. RAW264.7 cells were electroporated with the plasmid DNA. One of the stable transformants that showed highest response to LPS was isolated and used for the following experiments.

Cells were cultured in growth medium containing mAbs or other stimulants as indicated. The assays were carried out using the Picagene system (Wako), and a CT9000 luminometer (Dia-Iatron, Tokyo).

RT-PCR analysis of G-CSF gene expression in peritoneal macrophages

The cells at 1.5×10^6 cells/mL were cultured for 18 h either in the presence (10–40 μg/mL) or absence of 3-4H7 mAb, and washed with PBS. Total RNA was extracted with acid guanidium thiocyanate and phenol/chloroform as described previously [33]. Detection of G-CSF mRNA contents was performed by the quantitative PCR method [34]. Briefly, 1 μg of each total RNA was allowed to form first-strand cDNA in a 33-μL reaction mixture using $oligo(dT)_{12-18}$ as a primer and a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). From the reaction mixture, for PCR of β-actin, 1 μL of cDNA solution was used directly for PCR, whereas for PCR of G-CSF, 5 μL was used. The reaction mixture contained 10 pmol of specific primers for β-actin or 20 pmol for G-CSF, a final concentration of 200 μM of dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 1 mg/mL gelatin, and 1 unit Taq DNA polymerase (Takara, Kyoto) in a final volume of 50 μL. PCR was performed for 30 cycles under the following conditions: 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for extension with a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany). Ten μL of amplified cDNA products were electrophoresed in a 2% (w/v) agarose gel (Takara) and stained with ethidium bromide. We performed more than three independent experiments to confirm the reproducibility. Primers of G-CSF were synthesized, and the sequences of the primers were as follows: G-CSF sense, 5'-GCTGTGGCAAAGTGCACT-3' (position 121–138); G-CSF antisense, 5'-ATCTGCTGCCAGATGGTG-3' (position 520–537) [35]. Primers for β-actin were purchased from Clontech (Palo Alto, CA).

Flow cytometric analysis

The antigens were cytofluorometrically detected. After washing with PBS, 1×10^6 cells were treated on-ice for 30 min with 50 μL of PBS, 1% FBS, 0.02% NaN_3 (PBS/FBS/NaN_3 buffer) containing 40 μg/mL of 3-4H7 mAb or 40 μg/mL of purified mouse IgM as a negative control. The cells were then treated for 30 min with 50 μL of PBS/FBS/NaN_3 buffer containing 20 μg/mL of affinity-purified PE mouse anti-mouse IgM^a (PharMingen). After washing with PBS/FBS/NaN_3 buffer at 4°C, cells were fixed with 3.7% formaldehyde in PBS. For analysis of primary cells, cells were stained with both 20 μg/mL of FITC anti-lineage marker (CD3, CD14, and B220) mAb and with 40 μg/mL of 3-4H7 mAb, or with both FITC anti-lineage marker mAbs and the negative isotype

...e. These c... of PE-anti-mouse IgM[a] for 20 min. After washing with PBS/FBS/NaN_3 buffer at 4°C, the stained cells were fixed with 100 μL of 3.7% formaldehyde in PBS, and washed again with PBS/FBS/NaN_3 buffer.

Using an EPICS ALTRA flow cytometer and EXPO 2 software (Beckman-Coulter), flow cytometric analysis was performed. Based on the forward and side scatter profiles, a gate range was set around the whole cell population, and 20,000 events in this gate range were acquired for each sample. For the cell lines, the antigens were detected by the cell-surface PE anti-mouse IgM[a]. For the primary cells, a two-color analysis was performed. Positive cell populations detected by FITC anti-lineage marker mAbs were further analyzed by the cell-surface PE anti-mouse IgM[a]. Macrophages were identified by their positive staining for FITC-conjugated CD14 monitored in FL1 (log scale), B cells were identified by $B220^+$ and T cells were identified by $CD3^+$. Compensation was applied to correct for FITC emissions entering the FL2 channel and for PE emissions entering the FL1 channel.

Immunoblotting

For Western blotting, total cell lysates or plasma membrane fractions of the RAW264.7 cells (5 μg protein/lane) were subjected to 10% PAGE in the presence of SDS. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF, ProBlott™, PE Biosystems, Foster City, CA) sheet. The PVDF sheet was then pretreated with BlockAce (SnowBrand, Tokyo) for 1 h, washed by PBS containing 0.1% Tween 20 for 20 min, and reacted with 20 μg/mL of the purified 3-4H7 mAb or control IgM at room temperature. The reaction was detected by an alkaline phosphatase pNPP kit (BIO-RAD Laboratories, Hercules, CA).

RESULTS

Isolation of mAbs that stimulate G-CSF gene expression

We produced mAbs against the target cell surface and then tested their ability to stimulate G-CSF gene expression. The macrophage cell line we are interested in and the mouse strain conventionally used for immunization are isogenic, so using the normal BALB/c mouse strain would not raise an immune response against the antigens. Therefore, we used the autoimmune mouse (MRL/MPJ-*lpr/lpr*) and the normal rat (WKY) for hybridoma preparations. Immunization with whole cells from the macrophage cell line RAW264.7 resulted in increases in the titers against both strains, but it failed to boost the titer against the normal BALB/c mice, as expected (data not shown). As a result, we were able to obtain hybridomas from both MRL/MPJ-*lpr/lpr* mice and WKY rats. More than 6000 wells were screened for 7 MRL/MPJ-*lpr/lpr* mice and more than 5000 wells were screened for 4 WKY rats (**Table 1**). Of the approximately 12,000 wells examined, a total of more than 400 wells reacted positively to the RAW264.7 cells by ELISA. We cloned monoclonal hybridomas from 58 wells that showed strong responses. mAbs were purified from these hybridomas and their ability to stimulate G-CSF gene expression in RAW264.7 cells was examined with the reporter gene assay using luciferase. Seven mAbs from the MRL/MPJ-*lpr/lpr* mice (three IgG and four IgM) were found to increase the expression of G-CSF gene by factors of 6 to 40, but none from the WKY rats induced G-CSF gene in the RAW264.7 cells. The mAb that showed the highest stimulatory activity, 3-4H7, was chosen for further detailed examination.

TABLE 1. Summary of the Hybridoma Screenings

Animals[a]	Number of hybridoma wells examined	Number of ELISA-positive wells	Number of hybridomas monocloned	Number of hybridomas having positive luciferase inducibility
MRL/MPJ-*lpr/lpr*	6,600	283	43	7
WKY	5,500	183	15	0

[a] Seven MRL/MPJ-*lpr/lpr* mice and four WKY rats were used.

Dose-dependency and time-course of mAb-induced G-CSF gene expression

Expression of G-CSF gene in RAW264.7 by the purified 3-4H7 mAb was examined at 18 h after addition of the mAb to the culture medium. Dose-dependent stimulation of G-CSF gene expression was observed at mAb concentrations ranging from 2.0 to 60 μg/mL. Thirty- to sixty-fold increases in expression occurred at concentrations in the range 30–60 μg/mL (**Fig. 1A**). Under identical conditions, LPS, a potent stimulatory factor for cytokines, at 100 ng/mL resulted in a 200- to 300-fold increase in expression (Fig. 1B). The time-course of induction was examined using 3-4H7 mAb at a concentration of 40 μg/mL. G-CSF gene expression by 3-4H7 mAb was initially observed at 2 h after the addition of mAb, rapidly increased up to 8 h, and then decreased in the following 16-h period (**Fig. 2**). Under identical conditions, LPS at 100 ng/mL was more effective in inducing G-CSF gene than 3-4H7 mAb, while its maximum stimulation was observed at 12 h, some hours after the maximum stimulation of 3-4H7 mAb.

Selective stimulation of G-CSF gene expression by mAb

The ability of the 3-4H7 mAb to stimulate G-CSF gene and other cytokines in RAW264.7 cells is shown in **Figure 3A**. The mAb stimulated G-CSF at least 10 times more than it stimulated four other cytokines (IL-1α, IL-1β, IL-6, and TNF-α?. In contrast, LPS (Fig. 3B) was much more effective than 3-4H7 in stimulating the four cytokines. LPS has its greatest stimulatory effect on IL-6, and decreasing stimulatory effects in the order TNF-α, G-CSF, IL-1α, and IL-1β. The adhesion molecule FN (Fig. 3C) stimulated G-CSF gene expression about twice as much as the 3-4H7 mAb, but it also stimulated TNF-α and IL-6 about as much as one-half to one-third of that of G-CSF. The adhesion molecule VN (Fig. 3D) was about as effective as FN in stimulating G-CSF, but it also stimulated TNF-α and IL-6 much more than did FN. Direct contact between RAW264.7 cells and cells of the pre-B leukemia cell line NFS-60 enhanced G-CSF gene expression (Fig. 3E), but the NFS-60 cells stimulated the expression of IL-6 as well, although to a lesser extent. These figures thus demonstrate that the specific stimulation of G-CSF gene expression by the 3-4H7 mAb was unique compared with the stimulations by the other stimulants.

mAb 3-4H7 stimulates expression of G-CSF mRNA in normal peritoneal macrophages

The mAb clearly triggered G-CSF mRNA expression in peritoneal macrophages from normal mice (**Fig. 4**). In addition,

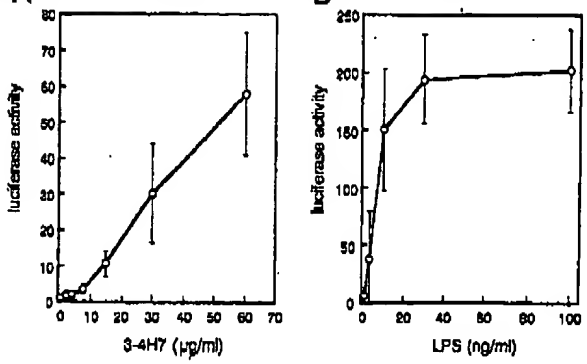

Fig. 1. Effect of various concentrations of 3-4H7 mAb or LPS on induction of G-CSF gene expression in RAW264.7 cells. RAW264.7 cl.27-3 cells were cultured with varying concentrations of 3-4H7 mAb (A) or LPS (B) for 18 h. After the treatments, the cells were lysed and assayed for luciferase activity. Data are shown as a mean of four experiments ± SE.

this effect was concentration-dependent for mAb concentrations in the range 10–40 μg/mL, which is consistent with the G-CSF gene expression observed using the luciferase reporter assay. These results show that the specificity of the mAb for G-CSF induction was not due to the choice of cell lines.

Flow cytometric detection of antigen(s) of the 3-4H7 mAb

A flow cytometric analysis was performed to determine whether the antigen(s) recognized by the 3-4H7 mAb is also unique to the RAW264.7 macrophage cell line or a property of all normal monocytes/macrophages, or found in other types of cells. We tested several murine cell lines, including monocytic/macrophage-like cell lines, myelocytic leukemia cell lines, and fibroblastic cell lines. In addition, we tested five human cell lines to determine whether they show cross-reactivity with this mAb. We also tested normal mouse peritoneal macrophages, splenocytic T and B cells, and bone marrow B cells. The results are summarized in **Table 2** and **Fig. 5, A–E**. These results demonstrated that the antigen(s) was found on the cell surface of not only the RAW264.7 cells but also other cell lines, although at weaker intensities. Positive results were obtained from the monocytes/macrophage-like cells and some undifferentiated promyelocytic leukemia cell lines, although none of the fibroblastic cell lines were positive for the antigen(s) recognized by this mAb. It is interesting to note that human promyelocytic leukemia (HL-60) cells were negative to this mAb when they were in an undifferentiated state or when they differentiated into granulocytic cells by N^6,2′-o-dibutyryladenosine 3′:5′-cyclic monophosphate (dbcAMP), but they were positive when they differentiated into monocytic cells by phorbol 12-myristate 13-acetate (PMA). Among the normal hematopoietic cells obtained from normal mice, only peritoneal macrophages reacted positively with the 3-4H7 mAb, whereas bone marrow B, spleen T, and spleen B cells were completely negative (Fig. 5, A–E).

Detection of antigen(s) by Western blotting

The above evidence strongly suggested that the antigen(s) recognized by this mAb reside on the cell membrane. To confirm this, we performed Western blotting against the whole-cell lysates of the RAW264.7 cells. A single band was identified at about 30 kDa in the Western blot with the 3-4H7 mAb, whereas no signals were found in the blot with the control mAb (**Fig. 6**). This band must correspond to an antigen that specifically bound to the mAb 3-4H7.

DISCUSSION

In this study, we demonstrate that a stimulatory mAb (3-4H7) selectively potentiates G-CSF gene expression in a mouse macrophage cell line, i.e., it does not measurably induce other inflammatory cytokines, such as IL-1α, IL-1β, IL-6, and TNF-α. Our success in obtaining these mAbs was due to two factors. One was to set up a reliable reporter gene assay system for quantitatively detecting G-CSF gene expression [23]. For this we used a cell line cloned from a macrophage cell line in which expression of G-CSF gene is especially sensitive to various stimuli, such as LPS. There have been no practical methods like ELISA to detect murine G-CSF because antibodies against murine G-CSF suitable for use in ELISA are not yet available. Therefore, as an alternative assay, a reporter gene assay was developed by introducing a fusion gene into three macrophage cell lines. The fusion gene has a promoter region from a mouse G-CSF gene juxtaposed to a luciferase gene at a 3′ downstream of the promoter [23]. Among the macrophage cell lines, the cell line RAW264.7 was found to be the best because it showed the lowest background and the highest signal of induced levels of G-CSF gene by several stimulants [23]. The other factor was the use of an autoimmune mouse strain for immunizations. We first immunized the commonly used BALB/c mice with this cell line, but the immunization failed to boost the titer in these mice. As a second choice, we used a rat strain (WKY) for immunization, hoping that a different species would show a greater immune reaction. Immunization of the rats with the cells successfully boosted the titer, but unfortunately, no stimulatory antibodies were recov-

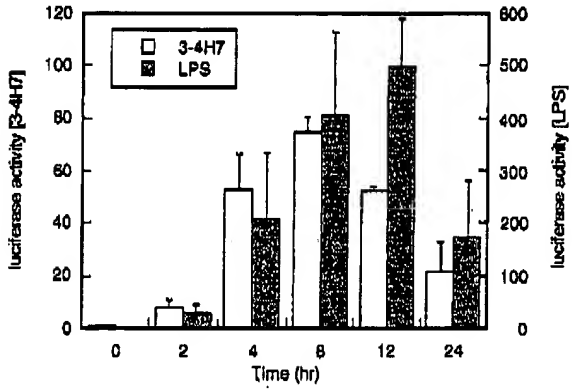

Fig. 2. Time-course of G-CSF gene expression by 3-4H7 mAb or LPS. RAW264.7 cl.27-3 cells were cultured for 0, 2, 4, 8, 12, and 24 h with 40 μg/mL of 3-4H7 mAb or 100 ng/mL of LPS. Luciferase activity was measured as described in Materials and Methods. Luciferase activity was expressed as described in Figure 1. Data are expressed as described in Figure 1 and shown as a mean of three experiments ± SE.

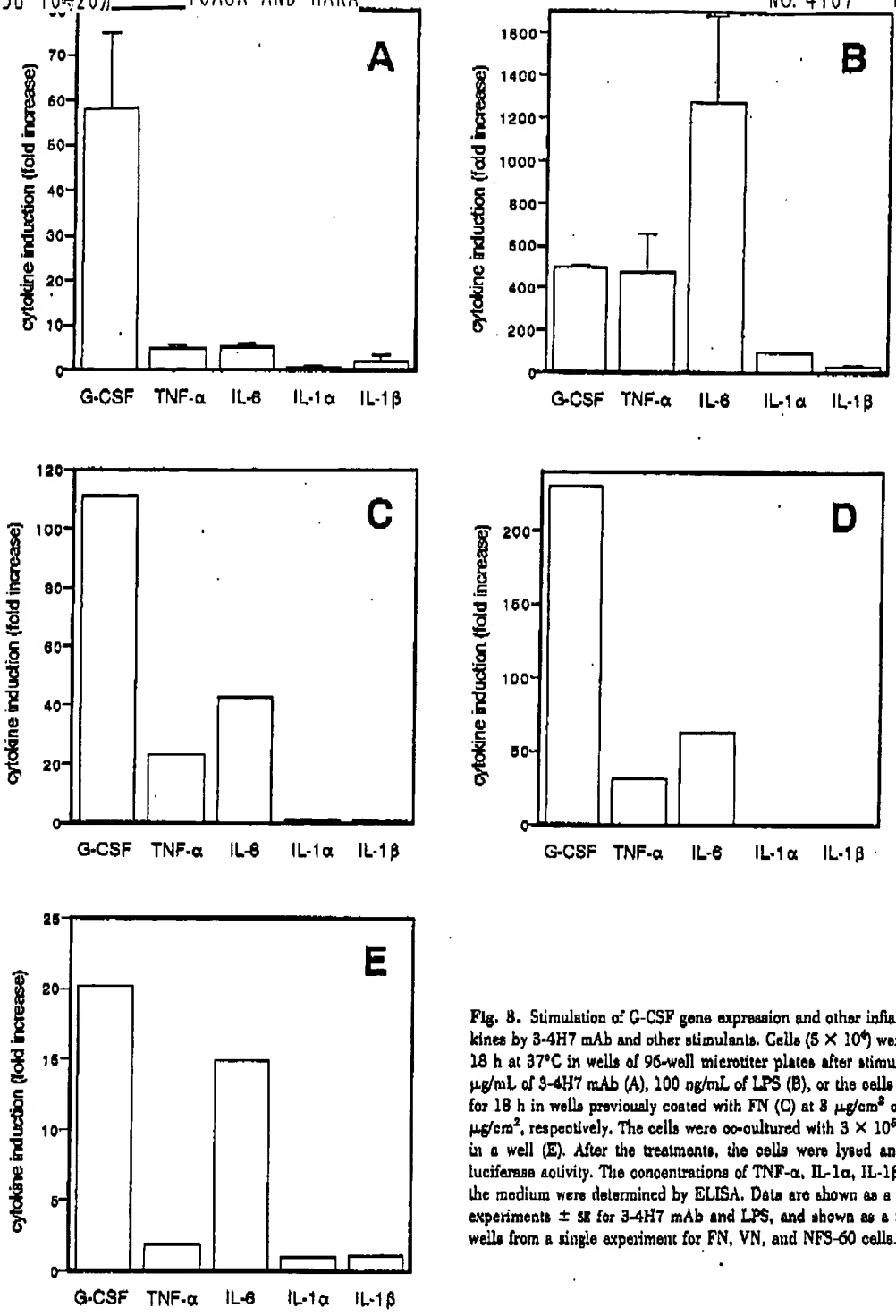

Fig. 8. Stimulation of G-CSF gene expression and other inflammatory cytokines by 3-4H7 mAb and other stimulants. Cells (5×10^6) were cultured for 18 h at 37°C in wells of 96-well microtiter plates after stimulation with 40 μg/mL of 3-4H7 mAb (A), 100 ng/mL of LPS (B), or the cells were cultured for 18 h in wells previously coated with FN (C) at 8 $\mu g/cm^2$ or VN (D) at 1 $\mu g/cm^2$, respectively. The cells were co-cultured with 3×10^5 NFS-60 cells in a well (E). After the treatments, the cells were lysed and assayed for luciferase activity. The concentrations of TNF-α, IL-1α, IL-1β, and IL-6 in the medium were determined by ELISA. Data are shown as a mean of three experiments ± SE for 3-4H7 mAb and LPS, and shown as a mean of three wells from a single experiment for FN, VN, and NFS-60 cells.

ered. As another alternative, we tried using an autoimmune mouse strain. We chose the MRL/MPJ-*lpr/lpr* strain only because we had previously succeeded in isolating catalytic antibodies from this strain in much greater quantities than we did with the conventionally used strain [36]. As we found in this study, this strain was also a good one for obtaining stimulatory antibodies.

In this study, we carefully checked, and ruled out, the possibility that trace contaminants in the medium were responsible for the expression of G-CSF gene. First of all, in order to minimize the possibility that trace elements in the serum were responsible for the expression of G-CSF gene, all antibody samples used for the assays were carefully isolated from the hybridomas by cultivating them in medium completely free of bovine serum. In our previous

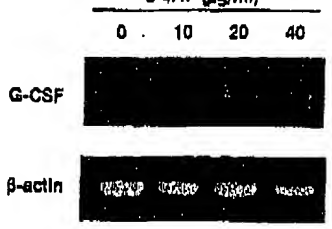

Fig. 4. RT-PCR analysis of G-CSF gene expression in primary peritoneal macrophages. Quantitative RT-PCR was performed as described in Materials and Methods. The size of the G-CSF transcripts measured was approximately 416 bp.

experiments, we found that LPS was a potent inducer of G-CSF gene expression at the nanogram per milliliter level [23], so contaminants like endotoxins may affect the results even at a trace level. Soluble factors such as cytokines also may trigger G-CSF gene expression even at a trace level. Trace levels of the cytokines IL-4, M-CSF, and oncostatin M might be important because low levels of these cytokines were found to induce G-CSF in monocytes/macrophages [13, 20, 21, 32]. To evaluate these possibilities, we examined how the expression of G-CSF gene depends on the concentration of the mAb, and determined whether there is difference in the time-course patterns among the stimulatory factors. The induction was dependent on the concentration of the mAb. The kinetics of induction by this mAb were different from the kinetics of induction by LPS in that the time of the maximum induction was earlier for the mAb than for LPS. In addition, we found that induction of G-CSF gene expression by LPS was not suppressed by the addition of the 3-4H7 mAb (data not shown). We also found that M-CSF was not responsible for the induction (data not shown), although we have not yet tested other stimulants.

TABLE 2. Flow Cytometric Analysis of the Distribution of Antigen(s) Recognized by the 3-4H7 mAb Among Various Cell Types

Cell	Cell type	Expression level[a]
Mouse cell line		
RAW264.7	Macrophage/monocyte	+++
J774.1	Macrophage-like	+
PU5-1.8	Macrophage/monocyte	±
M1	Myeloblast	−
WEHI-3	Myelomonocyte	±
P815	Mast cell	−
PAI	Lymphoblast	++
L5178Y	Lymphoblast	++
NFS-60[c]	Myeloblast	++
M-NFS-60[b]	Myeloblast	−
Mouse primary cell		
Peritoneal macrophages	Macrophage	++
Splenocytic B cells	B lymphocyte	−
Splenocytic T cells	T lymphocyte	−
Human cell line		
HL-60	Promyelocyte	−
HL-60 + PMA[c]	Macrophage-like	+
HL-60 + dboAMP[d]	Neutrophil-like	−
U937	Histiocyte	−
MOLT-4	T lymphoblastoid	−
K562	Lymphoblast	−
HeLa	Epithelial-like	±
Hamster cell line		
V79	Fibroblast	−
Monkey cell line		
COS-7	Fibroblast	−

Cells (10^6 cells) were treated with mAb 3-4H7 or anti-TNP IgM for 1 h on ice and reacted with PE-conjugated anti-mouse IgM[e]. The fluorescence intensity was analyzed using an EPICS ALTRA flow cytometer. [a] G-CSF-dependent cell line. [b] M-CSF-dependent cell line. [c] PMA was treated before the assay. [d] dboAMP was treated before the assay. [e] The mean value of fluorescence intensity of the tested sample vs. that of the reference control sample is: +++, >10.0; ++, 10.0–2.0; +, 2.0–1.5; ±, 1.5–1.25; and −, <1.25.

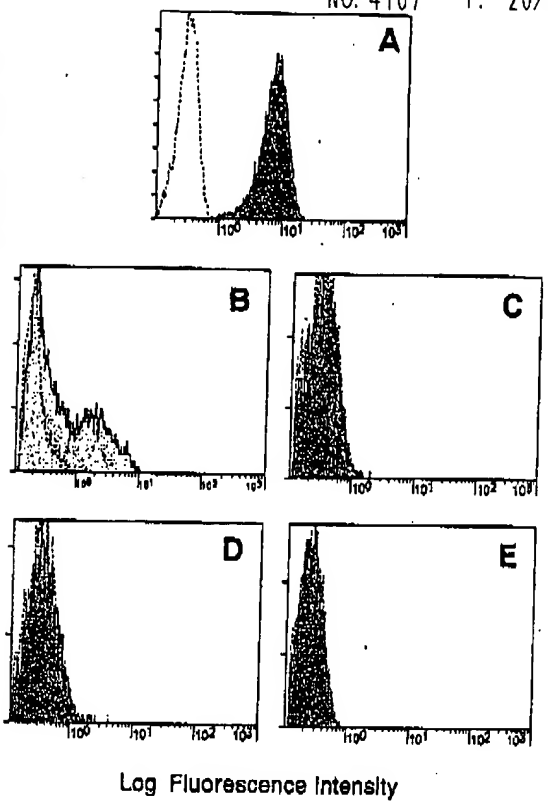

Fig. 5. FACS analysis of RAW264.7 cells and normal hematopoietic cells stained with 3-4H7 mAb. RAW 264.7 cells (A), and the macrophages from the peritoneal exudate (B), bone marrow B cells (C), splenic T cells (D), and splenic B cells (E) freshly isolated from the C57BL/6 mice were stained with 40 μg/mL of the 3-4H7 mAb and PE-conjugated anti-mouse IgM[e] and analyzed on an EPICS ALTRA flow cytometer (solid lines). Controls were stained with anti-TNP IgM and PE-conjugated anti-mouse IgM[e] (dashed lines). Histograms show the intensity of the fluorescence on a logarithmic scale (x-axis) and the relative cell number (y-axis), respectively.

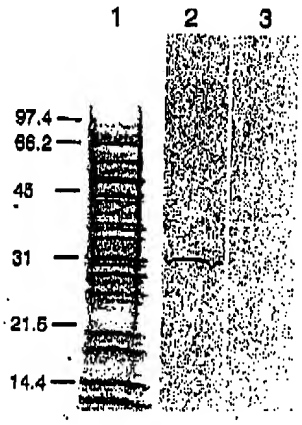

Fig. 6. Western blotting of antigen(s) on the RAW264.7 cells recognized by the 3-4H7 mAb. Lane 1, the gel was stained with Coomassie brilliant blue; lane 2, Western blotting with the 3-4H7 mAb; lane 3, Western blotting with the control IgM. Molecular weight standards are shown on the left.

The present data strongly suggested that the stimulatory effect was due to the mAb, and not to residual amounts of other contaminants. The 3-4H7 mAb did not stimulate the luciferase activity to the RAW264.7 cells, which were transfected with just a Picagene Enhancer Vector 2, but without the promoter region of G-CSF. In general, antibodies do not penetrate into the cells. Taken together, it was quite unlikely that the 3-4H7 mAb nonspecifically activated the luciferase. In addition, we have directly tested the trace amount of LPS in the 3-4H7 mAb samples through the use of an LPS-detecting kit (Seikagaku Kogyo, Tokyo). It was 7.23 pg/μg of the 3-4H7 mAb protein in the testing sample. So, the 3-4H7 mAb sample of 60 μg/mL should contain 432 pg/mL. As shown in Figure 1, the induction of luciferase at 1 ng/mL of LPS over the control was 6.26 ± 5.91. So the luciferase induction by LPS could be about a half of 6.26, whereas at this concentration induction of the luciferase was 57.8 ± 17.1. Therefore, contribution of LPS in the sample for luciferase induction could be around 5% at most. We thought that this level of contamination affected our results little, if at all.

An important finding of this study was that this mAb was selective in the induction of the cytokines. It induced G-CSF gene, but not other inflammatory cytokines, such as IL-1α, IL-1β, IL-6, and TNF-α. This result was quite different from the results obtained by ECM proteins that are known to induce G-CSF gene expression [22]. None of them selectively induced G-CSF gene expression, but they did show rather pleiotropic potency toward the induction of inflammatory cytokines. Based on these observations, we speculated that the signal pathway stimulated by the mAb was selective and not shared by the pathways stimulated by these other stimulants.

We then attempted to determine whether this phenomenon was specific to RAW264.7 cells or a relatively common property of macrophages. None of the methods for detecting G-CSF gene expression is better than the reporter gene assay, and only quantitative RT-PCR permitted us to measure G-CSF gene expression in normal tissues. We found that the mAb was also effective in stimulating G-CSF gene expression in the primary macrophages of the peritoneal exudate cells from the normal mice. This result suggested that a stimulatory signal for G-CSF gene expression via the mAb to the monocytic/macrophage lineage was more common than we expected. The signal or signals mediated by the mAb were blocked by Herbimycin A (data not shown). So, the tyrosine kinase cascade seems to be involved in induction of G-CSF gene expression. Whether these are novel molecules or molecules that have been previously described was of particular interest to us. Therefore, we tried to identify the molecule(s) through the use of flow-cytometric analysis. The results of this analysis demonstrated that this mAb was bound to the surface of RAW264.7 cells. Antigens were also found to be distributed in monocytic/macrophage cell lines, and some lymphoblastic and/or myeloblastic cell lines as well. An important finding was that the molecule(s) was distributed in the normal peritoneal macrophages, but it was not distributed in the normal T and B lymphocytes from the spleen or in the normal B lymphocytes from the bone marrow. In the case of the human promyelocytic leukemia cell line HL-60, the mAb cross-reacted with the molecule(s). It is interesting that a cross reaction was observed in the cells upon their differentiation into macrophage-like cells by PMA. The Western blotting indicated that the size of the molecule mainly recognized by this stimulatory mAb was about 30 kDa. We have recently cloned a candidate cDNA by expression cloning and now we are trying to verify the details. To the best of our knowledge, membrane-associated molecules involved in signaling with a molecular mass around this range have not yet been reported in monocytic/macrophage cells. These results, taken together, strongly suggested that there are some signal gateways on the macrophage cell surface that lead to the induction of G-CSF gene expression. Among them, there is a specific gateway on the cell membrane, through which the extracellular stimulatory signal is transmitted to the intracellular signal molecule(s) for induction of G-CSF gene expression.

G-CSF is a widely used therapeutic cytokine for boosting neutrophils in bone marrow transplant patients and in patients with neutropenia after treatment with anticancer agents [2, 3]. However, due to the high cost of these therapies, the requirement for subcutaneous or intravenous administration, and the potential side effects, such as medullary bone pain, a small-molecular-weight compound that could be delivered with a minimal cost and low side effects would be of clinical value. Fine et al. found a small synthetic compound that selectively induced G-CSF [24]. The target of this compound could be one or more intracellular signal molecules. The present results obtained with the mouse cells could easily be extended to search for human counterparts of the molecule(s) recognized by this mAb. Such an effort will facilitate the development of biologically active molecules, such as peptides or antibodies as well as small molecular weight molecules that specifically induce G-CSF in peripheral monocyte/macrophage cells.

ACKNOWLEDGMENTS

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(71)Applicant : JAPAN TOBACCO INC
(72)Inventor : AOKI RYOKO
HIRANO DAISUKE
NAKAMURA MOTONAO
NISHI YOSHISUKE

(54) ANTIBODY CONTAINING GRANULOCYTE COLONY-STIMULATING FACTOR INDUCTION ACTIVITY AND ITS MEDICINE

(57)Abstract:
PROBLEM TO BE SOLVED: To obtain the subject new antibody comprising an antibody having granulocyte colony stimulating factor(G-CSF) induction activity or its part, having G-CSF production ability, useful as a preventive, a therapeutic agent, etc., for adverse effects of anti-cancer agent, opportunistic infectious disease, etc.
SOLUTION: This antibody or its part has granulocyte colony stimulating factor (G-CSF) induction activity, G-CSF production ability dependently upon concentration, shows treating effects on neutropenia and neutropenia after bone marrow transplantation and apastic anemia and is useful for preventing and treating various infectious diseases including opportunistic infection, etc. The antibody is obtained by administering mouse macrophage cell strain RAW 264.7 as an immunization antigen to an MRL/1 pr mouse by intraperitoneal administration four times at intervals of 7 days, collecting a splenocyte after the final immunization, fusing the splenocyte with a mouse myeloma cell, selecting a strain to produce a macrophage cell binding antibody from the obtained hybridoma and culturing the strain.

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WPI Acc No: 1999-308102/199926
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New antibody having granulocyte colony stimulating factor inducing activity - useful for treating neutropenia
Patent Assignee: JAPAN TOBACCO INC (NISB)
Number of Countries: 001 Number of Patents: 001
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JP 11106400	A		8	C07K-016/28	

Abstract (Basic): JP 11106400 A

Whole or a part of an antibody having granulocyte colony stimulating factor (G-CSF) inducing activity is new especially monoclonal antibody produced by a hybridoma (FERM BP-6103). Also claimed are: (1) the monoclonal antibody producing hybridoma (FERM BP-6103); and (2) the antibody used for prevention and treatment of neutropenia.

USE - For treating neutropenia due to adverse reaction of anticancer agents or after bone marrow transplantation, and aplastic anemia.

Dwg. 0/2

Title Terms: NEW; ANTIBODY; GRANULOCYTE; COLONY; STIMULATING; FACTOR; INDUCE; ACTIVE; USEFUL; TREAT
Derwent Class: B04; D16
International Patent Class (Main): C07K-016/28
International Patent Class (Additional): A61K-039/395; C12N-005/10; C12N-015/02; C12P-021/08; C12R-001-91
File Segment: CPI
Manual Codes (CPI/A-N): B04-G21; B04-H04A; B14-F03; D05-H11A1; D05-H15
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01 M423 M710 M903 P812 Q233 V600 V611